Differentiation of Enteric Bacteria Using the Fox 3000 Electronic Nose

by

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Abstract:

The study's objective was to examine the viability of using the Fox 3000 electronic nose to provide a rapid method of differentiation of enteric bacteria, commonly referred to as "coliforms", on the basis of their odor. Two coliform organisms, *Escherichia coli* 363 and *Enterobacter liquefaciens*, were sampled in the experiment to see if differentiation could be achieved. After being inoculated with $5x10^3$ to $1.5x10^4$ organisms, broth cultures were incubated for twenty-four hours. Samples for electronic nose testing and plate counting were taken at zero, seven, and twenty-four hours. Principle component analysis and discriminant function analysis programs were used to graph the odor profiles created by the headspace gases produced by each bacterium and the broth blanks.

Keywords: enteric bacteria, electronic nose, differentiation, coliforms, odor

Introduction:

In the food industry, much importance is placed upon rapid methods of enteric bacteria differentiation. Some enterics can be differentiated on the basis of their metabolism. Such a group of bacteria that can be differentiated from the rest of the enteric bacteria is the coliform group. Coliform bacteria are Gram negative, facultative rods that ferment lactose to acid and gas within forty-eight hours (Hitchins *et al.*, 1992). They can serve as indicators of poor sanitation in processing plants, and differentiation within the coliform group is important to determine if any of them may be pathogenic, such as *Escherichia coli* O157:H7. Current differentiation of such organisms requires plating the organisms in question on expensive selective and differential agars, such as those produced by CHROMagar, which differentiate the organisms on the basis of the

color the colony produced when grown on the agar (CHROMagar, 1999). The official method for coliform detection, as seen in the *Compendium of Methods for the Microbiological Examination of Foods*, states that the organisms should be plated on violet red bile agar to test for the presence of coliforms in the sample. Then, differentiation must be achieved using differential agar, such as eosin methylene blue agar (Hitchins *et al.*, 1992).

Both of the previously mentioned ways to differentiate coliforms would require forty-eight hours growth at 37°C before they could be examined to find the identity of the contaminating organisms. Therefore, a method that could differentiate the coliforms in less than forty-eight hours would be desired. While most coliforms have similar cellular and colonial morphologies, each genus produces a slightly different odor. Some may produce odors similar to those associated with fecal material while others may produce odors similar to those associated with decaying matter in soil. Identification and quantification of headspace volatiles composing these odors could prove useful in the identification of the organism.

A tool recently developed could aid in the differentiation of bacteria on the basis of their odor. The electronic nose has been gaining much exposure in the food industry as a useful quality control instrument. Its uses have ranged from fermentation control to fish inspection, so one would find it logical that the electronic nose could prove a useful tool to differentiate genera of coliforms on the basis of their gases produced (Techbrief, 1999).

The electronic nose is able to differentiate odors by using an array of sensors. The sensors may be composed of metal oxides, quartz resonator sensor arrays, and

conducting polymers (Bartlett *et al.*, 1997). As different volatile compounds come into contact with the metal oxide sensors, the conductivities of the sensors are changed such that the electric current passing through the sensor is altered. The alteration in the electric current is registered by the electronic nose to create a graphical profile for a compound. The amount of change in the sensor's conductivity is directly proportional to the type and concentration of compound coming into contact with that sensor. The quartz resonator sensor arrays work in a similar fashion to the metal oxide sensors, except the quartz resonates when the compound adsorbs to it. Being a piezoelectric material, the quartz can create changes in electric current when the compound adsorbs and desorbs. The change in electric current can be used to create the odor profile. The conducting polymer sensors are composed of polypyrroles and polyanilines and change in conductivity when in the presence of different volatile compounds. Once again these changes can be used to create the odor profile. The usefulness of the conducting polymer sensors would be in situations where the compounds to be tested reside in the 0.1 to 100 ppm range (Bartlett *et al.*, 1997).

Several researchers have already tried to use the electronic nose's vast array of sensors to detect and differentiate microorganisms. The Warwick-Southhampton Electronic Nose Group has already employed the electronic nose to differentiate between *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*. The study only proved moderately successful, however, as a discriminant function analysis showed good differentiation between *S. pyogenes* and *P. aeruginosa*, but poor differentiation between *S. aureus* and *E. coli*, as shown in figure 1 (Warwick-Southhampton, 1999).

The Warwick-Southhampton Electronic Nose Group also has investigated the use of the electronic nose to predict bacteria type and growth phase. In this experiment, they were able to train a neural network program so that *S. aureus* and *E. coli* could be differentiated and their growth phase predicted with a high degree of precision (Gardner *et al.*, 1997).

Additional research has been performed to differentiate a single species of a bacterium from itself on the basis of the number of viable organisms in a sample. Alpha M.O.S. conducted research with its version of the electronic nose, the Fox 4000, to find that samples of *E. coli* could be separated on the basis of number of organisms present using a principle component analysis (Lucas and Benicasa, 1998).

In a direct application of the electronic nose to the food industry, Judy W. Arnold of the Russell Research Center used the electronic nose to test for bacterial contamination in a poultry processing plant. Her data indicated that enteric bacteria from the poultry carcasses could successfully be differentiated by the electronic nose if the bacteria were in liquid samples. Arnold also found that numbers of bacteria could be determined after training a neural network program to identify the organism and its number (Arnold, 1997).

Materials:

1 – pure culture of *Escherichia coli* 363

1 – pure culture of *Enterobacter liquefaciens*

2 – agar slants (BBL Trypticase Soy Agar)

38 – 10 mL dilution tubes with 9 mL sterilized distilled water

2 – 10 mL tubes of Difco Tryptose Broth

vortexing machine

26°C incubator

4°C refrigerator

Bunsen burner

metal loop

1 mL Eppendorf pipette

1 mL Fisherbrand Redi-tips

2 – 250 mL Erlenmyer flasks filled with 99 mL of Difco Tryptose Broth

1 – 250 mL Erlenmyer flasks filled with 150 mL of Difco Plate Count Agar

16 – 1.5 mL sterilized Fisherbrand Flat Top Microcentrifuge Tubes

6 – 100*15 mm Fisherbrand Petri Dishes

22 – 10 mL electronic nose sample vials

22 – clampable 20 mm metal caps with septums

Procedure:

Culture and Sample Preparation:

In order to test the usefulness of the electronic nose to differentiate coliforms, two organisms were chosen for comparison. Pure broth cultures of *E. coli* 363 and *E. liquefaciens* were obtained. Agar slants were made using BBL Trypticase Soy Agar to hold the bacteria for the duration of the experiment. The pure culture tubes were vortexed, and using aseptic technique, the bacteria were streaked across the surface of the agar slants using a sterile loop. The slants were incubated at 26°C for forty-eight hours to allow adequate growth. They were then refrigerated at 4°C until needed.

After removing the agar slants from refrigeration, a sterile loop was used to obtain some cells from the agar slants to be used to inoculate 10 mL of Difco Tryptose Broth. The cells were aseptically transferred to the tube containing the broth, and the broth was vortexed and then incubated at 26°C for twenty-four hours.

After incubation, each culture tube was vortexed and a series of three 9 mL sterilized distilled water dilution tubes set up for each culture broth. Using a 1 mL Eppendorf pipette and disposable Fisherbrand 1 mL Redi-tips, 1 mL from each culture broth was transferred to the first tube of 9 mL of sterilized distilled water. The dilution tube was then vortexed and the dilution was continued through the other two dilution tubes with adequate vortexing between each step. The dilution factor at this point was 10^{-3}. A final dilution was made into 99 mL of sterilized Difco Tryptose Broth in a 250 mL Erlenmyer flask bringing the overall dilution factor to 10^{-5}. After adequate shaking to mix the inoculated flask, the number of colony formation units per milliliter of broth culture (CFU/mL) in each 100 mL broth culture should be close to 10^4 CFU/mL.

From the 100 mL broth culture (in the flask), 1 mL was pipetted to each of three 10 mL electronic nose sample vials, such that triplicates of each bacterium could be obtained at each specified time. The vials were capped using 20 mm crimpable metal caps with septum in their centers. This first round of additions to the sample vials represented zero hour, and the vials were stored at 4°C until all samples for the experiment had been collected. Plate counts were also performed at zero hour using 1 mL from the 100 mL broth and diluting it with two 9 mL sterilized distilled water dilution tubes before adding the organism to the plate with 10 mL of Difco Plate Count

Agar. In addition to inoculated broth samples, 1 mL of a sterilized uninoculated broth was aseptically transferred into each of three 10 mL electronic nose sample vials.

In order to obtain cultures at seven and twenty-four hours, 1 mL of each organism's 100 mL inoculated broth (from the flask) was transferred to eight 1.5 mL Fisherbrand Flat Top Microcentrifuge Tubes. The tubes were incubated in a Cobas EIA vibrating incubator set at 37°C. After seven hours of incubation, three of each culture's centrifuge tubes were transferred to electronic nose sample vials, capped, and stored at 4°C. In addition, one of each culture's centrifuge tubes was diluted through a series of seven 9 mL sterilized distilled water dilution tubes in order to facilitate a plate count of each bacterium at seven hours. In a similar fashion, the remaining tubes were taken from incubation at twenty-four hours and added to sample vials and plated after seven dilutions. The plates at all three times were counted after 36 hours incubation at 26°C.

Electronic Nose:

The electronic nose system used for testing the headspace gases of each bacterium was the Fox 3000 produced by Alpha M.O.S. The nose was equipped with 12 metal oxide sensors for headspace gas analysis. Inoculated broth cultures were added to 10 mL sample vials in 1 mL amounts for headspace sampling. All samples to be tested were analyzed in triplicate.

After the samples were prepared, the air was turned on for the system and the system was allowed to equilibriate for ninety minutes before testing started. While the system was equilibriating, the set up for the experiment was established. First of all, the autosampler was set to heat each sample to 40°C before withdrawal of headspace volatiles for analysis. The acquisition time for the sensors was 120 seconds; the flow rate

was 250 mL/minute; and the delay between sample injections was 300 seconds. When the system was ready, twenty-one samples were removed from 4°C storage to be tested. The samples totaled twenty-two and included: one air blank (not refrigerated), three broth blanks, nine *E. coli* 363 samples, and nine *E. liquefaciens* samples.

At the conclusion of the data collection by the electronic nose, the data was examined with principle component analysis and discriminant function analysis by the computer program provided with the Fox 3000 Electronic Nose.

Results:

Preliminary Testing:

Due to the fact that a concrete method for the differentiation of enteric bacteria had not been published prior to the inception of the experiment, much initial experimentation was necessary to determine the medium of choice in which to culture the bacteria. A concern with differentiating the bacteria using the electronic nose was that the broth used to grow the bacteria would interfere with the interpretation of the headspace by the electronic nose. A simple solution of glucose and water was the first choice of a medium. After inoculation from an agar slant, both organisms survived in the solution after twenty-four hours, but inoculations from that glucose solution into other glucose solutions would not allow growth of *Enterobacter liquefaciens*, and the turbidity of *Escherichia coli* 363 cultures declined with subsequent transfers to glucose solutions. The reason for the lack of growth was determined to be the lack of a nitrogen source in the glucose solutions. Any nitrogen or protein present from the initial inoculum was only enough to provide for the growth of the organisms for twenty-four hours. Since growth

could not be maintained, no electronic nose testing was performed on the cultures grown in glucose tubes.

After the experiment with using a very simple medium failed, a more complex medium was chosen despite the consequences it may have had to the electronic nose sensor output. Trypticase Soy Broth (TSB) was chosen for its rich sources of protein and glucose that would eliminate the growth problems previously experienced. In order to obtain samples for electronic nose testing, 4 mL of sterile broth was transferred to each of nine 10 mL electronic nose sample vials. From previously inoculated and incubated TSB cultures, each organism was transferred by sterile loop to three vials so it could be tested in triplicate. All sample vials were capped to trap headspace volatiles and incubated at 37°C for twenty-four hours before testing. Before testing began, each sample was heated to 40°C to evolve more volatile compounds into the headspace. After testing, the resulting principle component analysis (PCA), shown in figure 2, showed that good differentiation could be made between the uninoculated broth and the organisms, thus eliminating the concern of the organisms' volatiles being overpowered by the broth's volatiles. Unfortunately, the PCA also showed that there was virtually no difference between the two organisms. When the vials were uncapped, gas could be heard escaping the vials. Massive carbon dioxide production due to glucose fermentation was hypothesized to be the reason for the lack of differentiation since the carbon dioxide could have conceivably blocked many of the other gases and volatile compounds in the headspace from reaching the sensors if it existed in a high concentration. Therefore, the procedure for growing the organisms before testing needed to be modified.

In order to alleviate the pressure of the carbon dioxide inside the sample vials, the organisms were incubated at 37°C for twenty-four hours in TSB tubes that were loosely capped instead of in the tightly capped sample vials. By growing the organisms in loosely capped tubes, the carbon dioxide could escape. From each tube was taken 1 mL of broth culture to be added to a 10 mL sample vial and capped. As always, each organism was examined in triplicate and the uninoculated broth was used as the control sample. Each sample was heated to 40°C before sampling. The results of the test showed on PCA, as shown in figure 3, that the organisms could be differentiated from the broth blanks and each other with a high degree of certainty. A discriminate function analysis (DFA), shown in figure 4, of the same data also showed excellent discrimination between the samples.

After an effective method had been determined for organism differentiation on the basis of odor, experimentation began on counting the organism. Many tests were performed with plate counting to determine the exact number of dilutions needed to obtain countable numbers of organisms on Plate Count Agar. Also tests were performed to determine the number of dilutions necessary to obtain an initial inoculum of 10^3-10^4 CFU/mL. An inoculum at that level would be representative of those found in the meat processing industry. All of the final determinations for number of dilutions needed at each point of the method could be seen in the procedure portion of the text.

Due to logistical problems, the 37°C incubator used to incubate the inoculated TSB tubes was no longer available. A smaller 37°C incubator was used in its place, and the procedure had to be modified to accommodate the change in incubators. Thus, the tubes previously used (20 mL capacity) had to be scrapped in favor of 1.5 mL

microcentrifuge tubes that would fit in the incubator. The change in culture container during incubation was most likely the cause for any discrepancy between final results and promising preliminary results.

Plate Counts:

An examination of the data obtained from several rounds of testing, following the procedure described in the procedure portion of the text, showed expected results for the plate counts, as shown in table 1. The target inoculum was 10^3-10^4 CFU/mL, and in all plates counted, the initial inoculum was in the target range. Both organisms were also enumerated at seven and twenty-four hours, ranging from $1.0x10^8$ CFU/mL to $9.8x10^8$ CFU/mL at seven hours and from $1.0x10^9$ CFU/mL to $6.6x10^9$ CFU/mL at twenty-four hours.

Multivariate Analysis:

Due to the relatively small inoculum of bacteria, one would have expected that not much differentiation would be seen between either organism and the broth at zero hours of growth when examining the results of electronic nose testing by multivariate analysis. A principle component analysis (PCA) of the results for zero hours growth, as shown in figure 5, showed no differentiation between the broth and the two organisms. A discriminant function analysis (DFA) of the data, as shown in figure 6, showed slightly better separation of the two organisms from the broth but not from each other.

Based on plate counts alone, one would not expect much difference between the multivariate analysis of results obtained at seven and twenty-four hours since both had similar numbers of organisms present. After examining the results at seven hours growth, one could see via PCA, as shown in figure 7, a differentiation beginning to occur

between the organisms and the broth; however, the data for the two organisms were still firmly placing the bacteria on top of each other. A DFA of the data at seven hours, as shown in figure 8, showed good separation between the two organisms and the broth and separation beginning to occur between the organisms themselves.

After examining the results at twenty-four hours growth, principle component analysis, as shown in figure 9, indicated that the two organisms were indeed separate organisms. An unexpected problem surfaced at twenty-four hours, though. While *E. liquefaciens* occupied its own region of the graph, many of the samples of *E. coli* 363 overlapped with the region occupied by the broth blanks, thus indicating no difference in the headspace volatile compounds. A discriminant function analysis, as shown in figure 10, indicated that there were in fact three distinct groups present.

Hypotheses and Conclusions:

Since a moderate differentiation between the organisms could be achieved at twenty-four hours using the electronic nose, a literature search was performed to find what the differentiating compounds might be between *E. coli* 363 and *E. liquefaciens.* The literature search indicated that *E. coli* 363 would produce metabolites, such as acetaldehyde, acids such as pyruvate, lactate, and acetate, ethanol, carbon dioxide, and hydrogen. *E. liquefaciens* produces many of the same compounds; however, it produces less acetate and hydrogen and vastly greater amounts of carbon dioxide, 2,3-butanediol, and ethanol (Gottschalk, 1986). The key component in all of the compounds mentioned would have to be 2,3-butanediol. This compound would be highly volatile, and *E. coli* 363 would produce virtually none of it.

If 2,3-butanediol was in fact the compound causing the greatest discrimination between *E. coli* 363 and *E. liquefaciens*, one would expect that the metal oxide sensors used by the Fox 3000 would register the highest discrimination for sensors affected by polar organic solvents, alcohols, and hydrocarbons (Fox 3000 Manual). A look at the discrimination power of the sensors shows this very trend, as is shown in table 2.

So all of this begs the question: why hasn't differentiation been achieved in the principle component analysis between *E. coli* 363 and the blank broth? One possible explanation could be that relatively large amounts of acetaldehyde and carbon dioxide were produced during Maillard browning and subsequent Strecker degradation brought on by autoclaving the broth blanks to sterilize them. If acetaldehyde and carbon dioxide compose the largest portion of the headspace above an *E. coli* 363 sample, the electronic nose may calculate during multivariate analysis that the sample is the same as an autoclaved broth sample, even though the human nose can distinguish between the two.

If the research were to continue, one would first want to cool the electronic nose autosampler to 4°-6°C to slow the growth of the bacteria and limit the drift of signals produced by bacteria when examined by PCA and DFA. In addition, one would want to examine the hours between seven and twenty-four hours to determine if the best differentiation could be achieved in that time period. One would also find it useful to examine the headspace volatiles by gas chromatography and mass spectrometry to obtain a clearer picture of the actual compositions of the odors. In addition, one would also want to expand the testing to include different species within the same genus to see if differentiation could be achieved at the species level and several more genera of enteric bacteria. The ultimate application of this research would be to actual food products to see

if the profiles of each bacterium obtained through testing could be used to identify unknown bacteria in foods.

In order for the electronic nose to be noticed by the food industry, the entire differentiation and identification process should take no more than twelve hours. In that time, the food should be sampled, incubated for 7-10 hours, and tested by electronic nose, such that a PCA and DFA can be obtained for comparison to standard samples of known bacteria.

References:

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Table 1. Enumeration of *E. coli* 363 and *E. liquefaciens* by pour plate method with plate count agar at the times the organisms were sampled by the electronic nose.

Organism	Trial #	0 hr.	7 hr.	24 hr.
Escherichia coli 363	Trial 1	$7.0x10^3$	$6.5x10^8$	$9.0x10^8$
	Trial 2	$1.0x10^4$	$5.6x10^8$	$1.0x10^9$
	Trial 3	$8.5x10^3$	$8.4x10^8$	$1.9x10^9$
	Trial 4	$7.0x10^3$	$9.8x10^8$	$1.0x10^9$
	Avg.	**$8.1x10^3$**	**$7.9x10^8$**	**$1.2x10^9$**
Enterobacter liquefaciens	Trial 1	$8.0x10^3$	$6.5x10^8$	$1.0x10^9$
	Trial 2	$7.0x10^3$	$5.6x10^8$	$2.0x10^9$
	Trial 3	$7.8x10^3$	$1.0x10^8$	$1.5x10^9$
	Trial 4	$8.1x10^3$	$4.0x10^8$	$6.6x10^9$
	Avg.	**$7.7x10^3$**	**$4.3x10^8$**	**$2.8x10^9$**

- results reported as CFU/mL

Table 2. Examination of discrimination power and compounds associated with the Fox 3000 metal oxide sensors after bacterial growth of twenty-four hours using the final procedure.

Sensor name	Discrimination Power	Associated volatiles
PA2	42.1	Organic solvents / polar compounds
T70/2	40.4	Aromatic compounds / alcohols
P10/2	40.1	hydrocarbons
P40/1	39.7	Aldehydes / fluoride, chloride
P10/1	37.4	Non polar hydrocarbons
SY/W	34.7	No specific compounds listed
T30/1	33.9	Organic solvents / polar compounds
SY/LG	33.4	Fluoride, chloride
SY/cG	32.1	No specific compounds listed
SY/gW	29.4	No specific compounds listed
SY/G	26.7	No specific compounds listed
SY/gCT	4.1	Nonpolar volatiles

- Information on sensors obtained from Fox 3000 manual

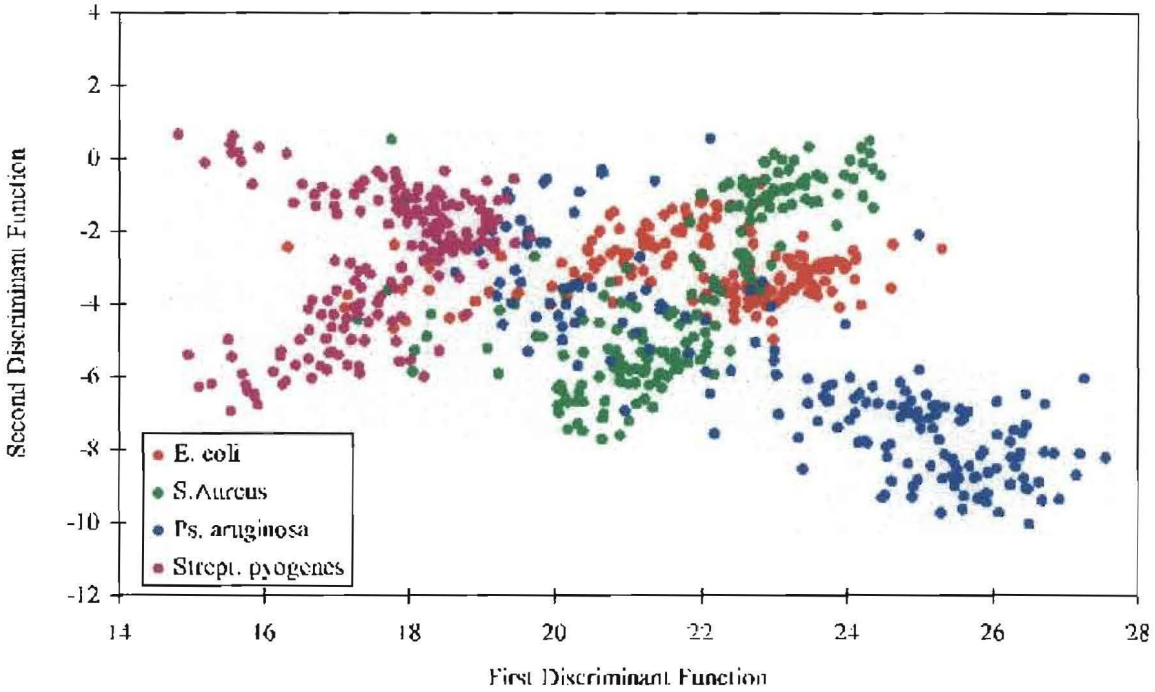


FIGURE 1. EXAMINATION OF SEVERAL ORGANISMS TO ACHIEVE DIFFERENTIATION USING DISCRIMINANT FUNCTION ANALYSIS.

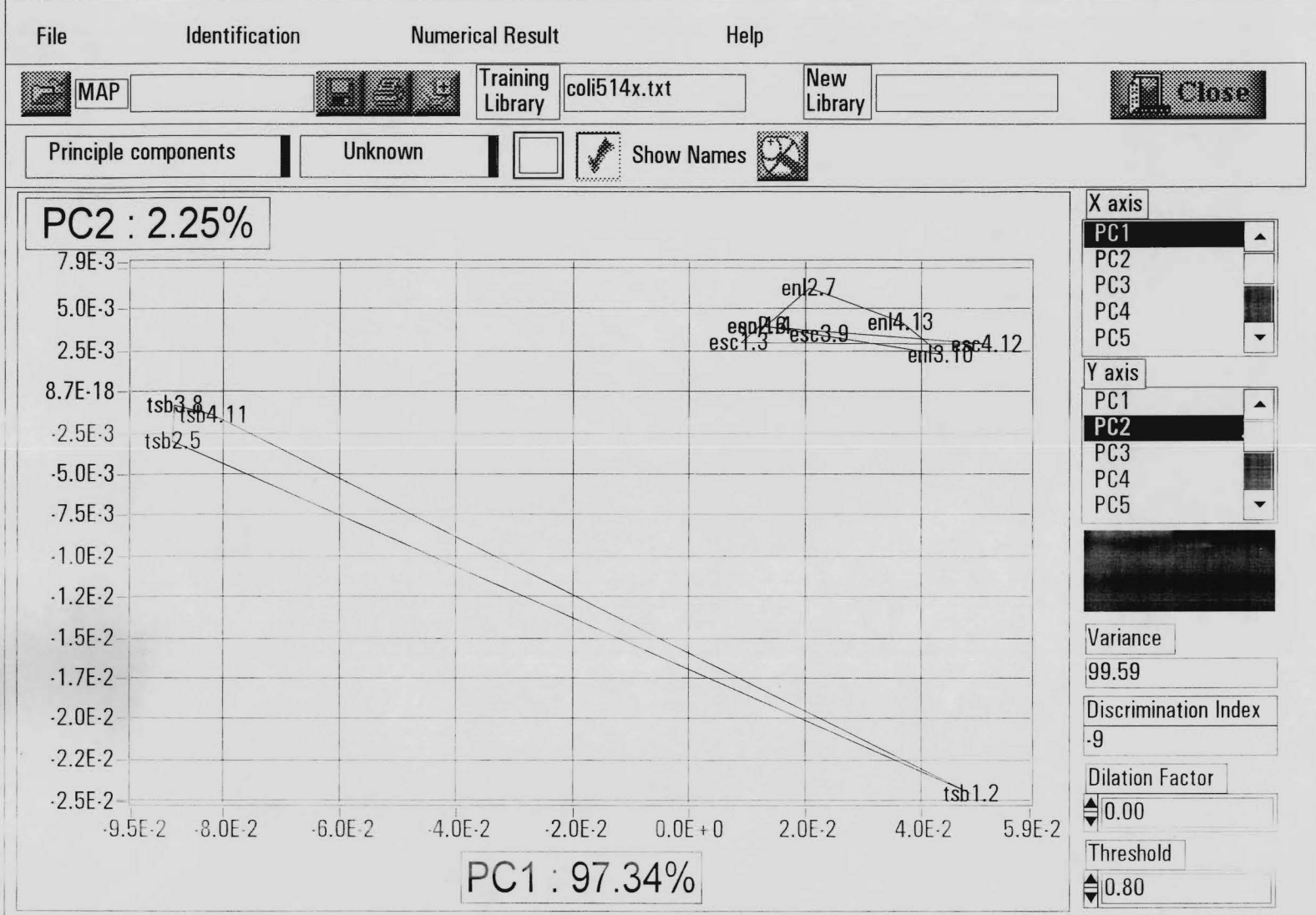


FIGURE 2. PRELIMINARY STUDY OF E. coli 363 AND E. liquefaciens EXAMINED BY PRINCIPLE COMPONENT ANALYSIS AFTER 24 HOURS GROWTH IN CAPPED SAMPLE VIALS.

- esc = E. coli 363
- enl = E. liquefaciens
- tsb = TRYPTICASE SOY BROTH

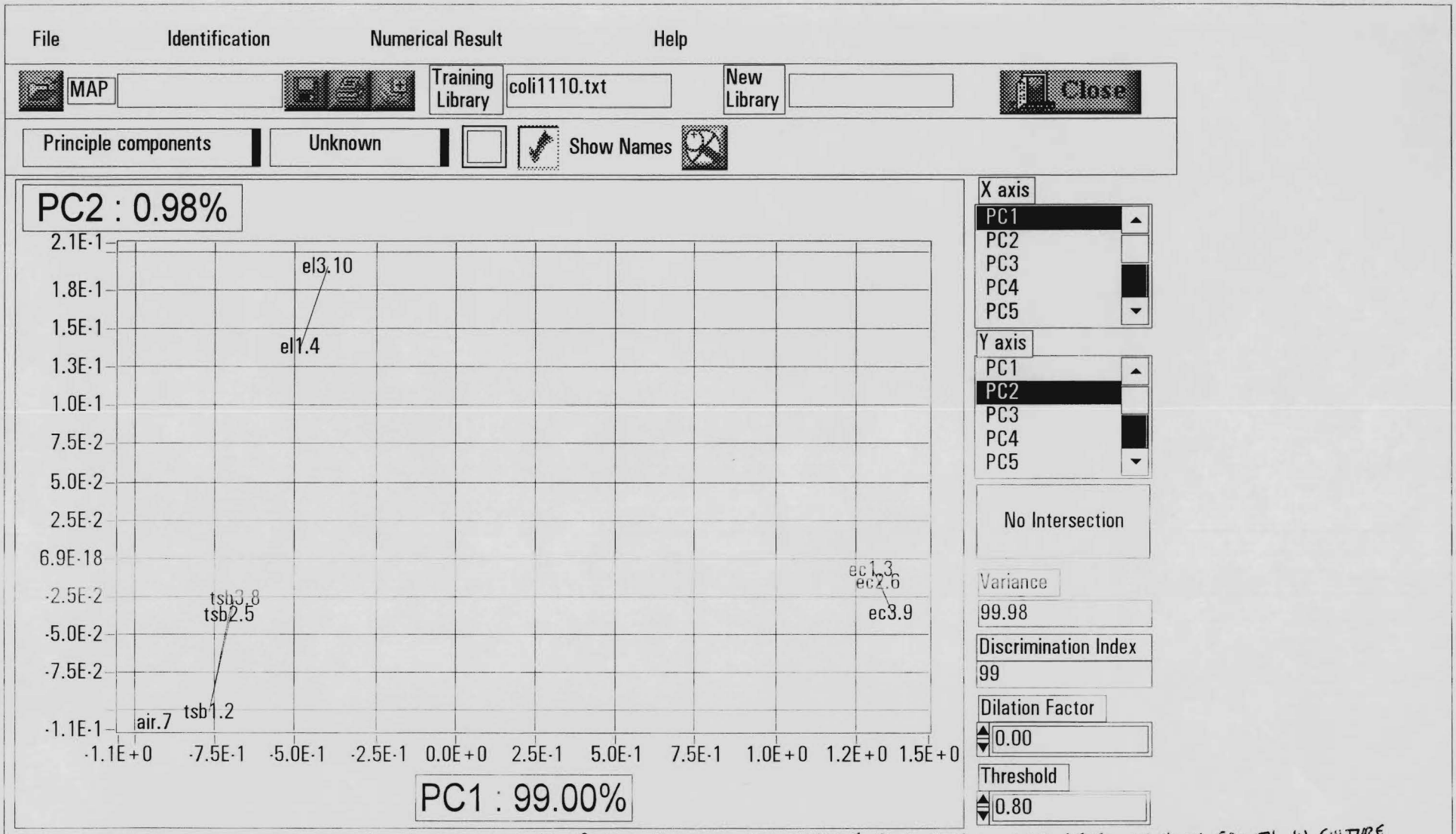


FIGURE 3. PRELIMINARY STUDY OF E. coli 363 AND E. liquefaciens EXAMINED BY PRINCIPLE COMPONENT ANALYSIS AFTER 24 HOURS GROWTH IN CULTURE TUBE.

- ec = E. coli 363
- el = E. liquefaciens
- tsb = TRYPTOSE BROTH

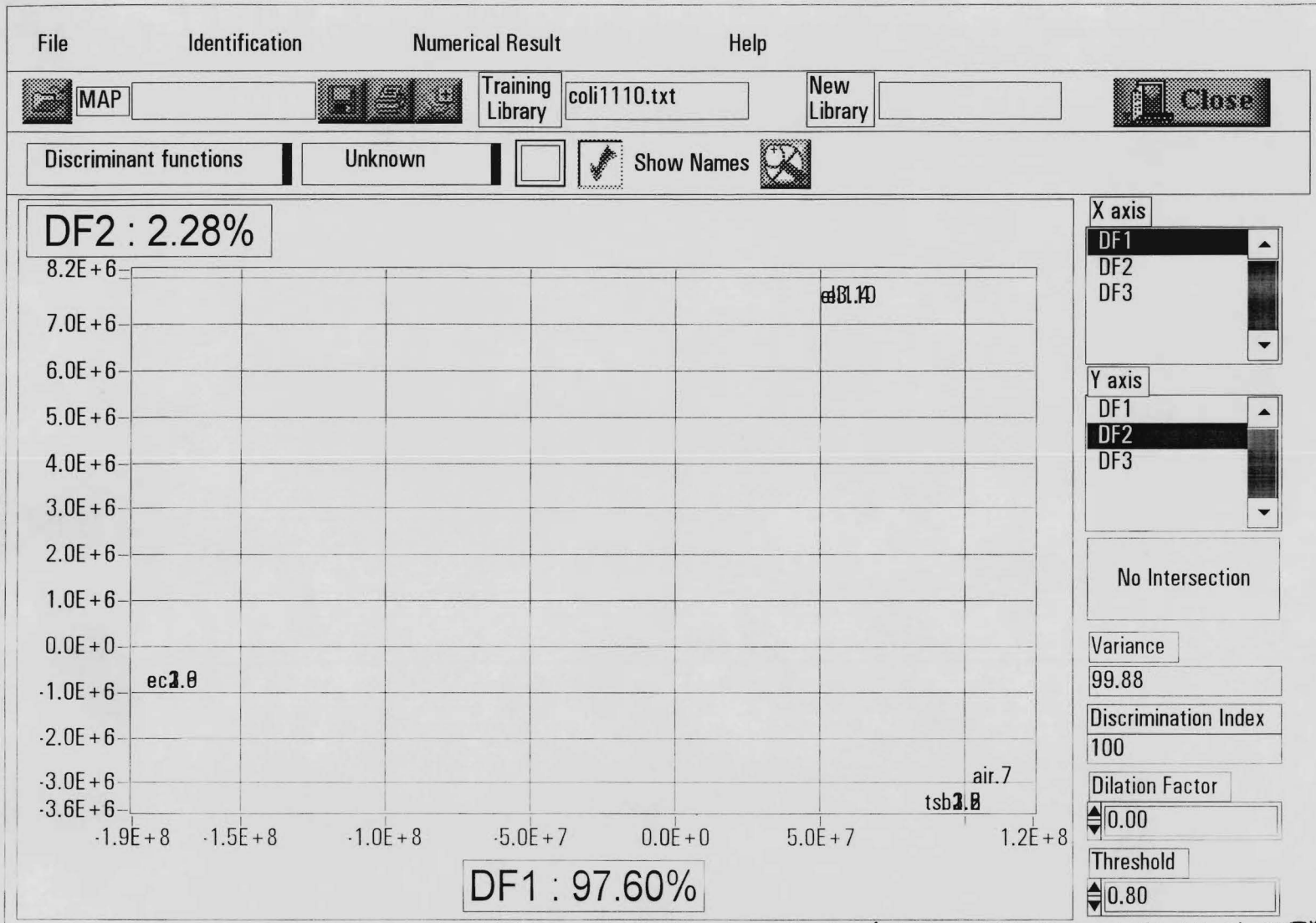


FIGURE 4. DISCRIMINANT FUNCTION ANALYSIS OF E. coli 363 AND E. liquefaciens AFTER 24 HOURS GROWTH IN CULTURE TUBES.

- ec = E. coli 363
- el = E. liquefaciens
- tsb = TRYPTOSE BROTH

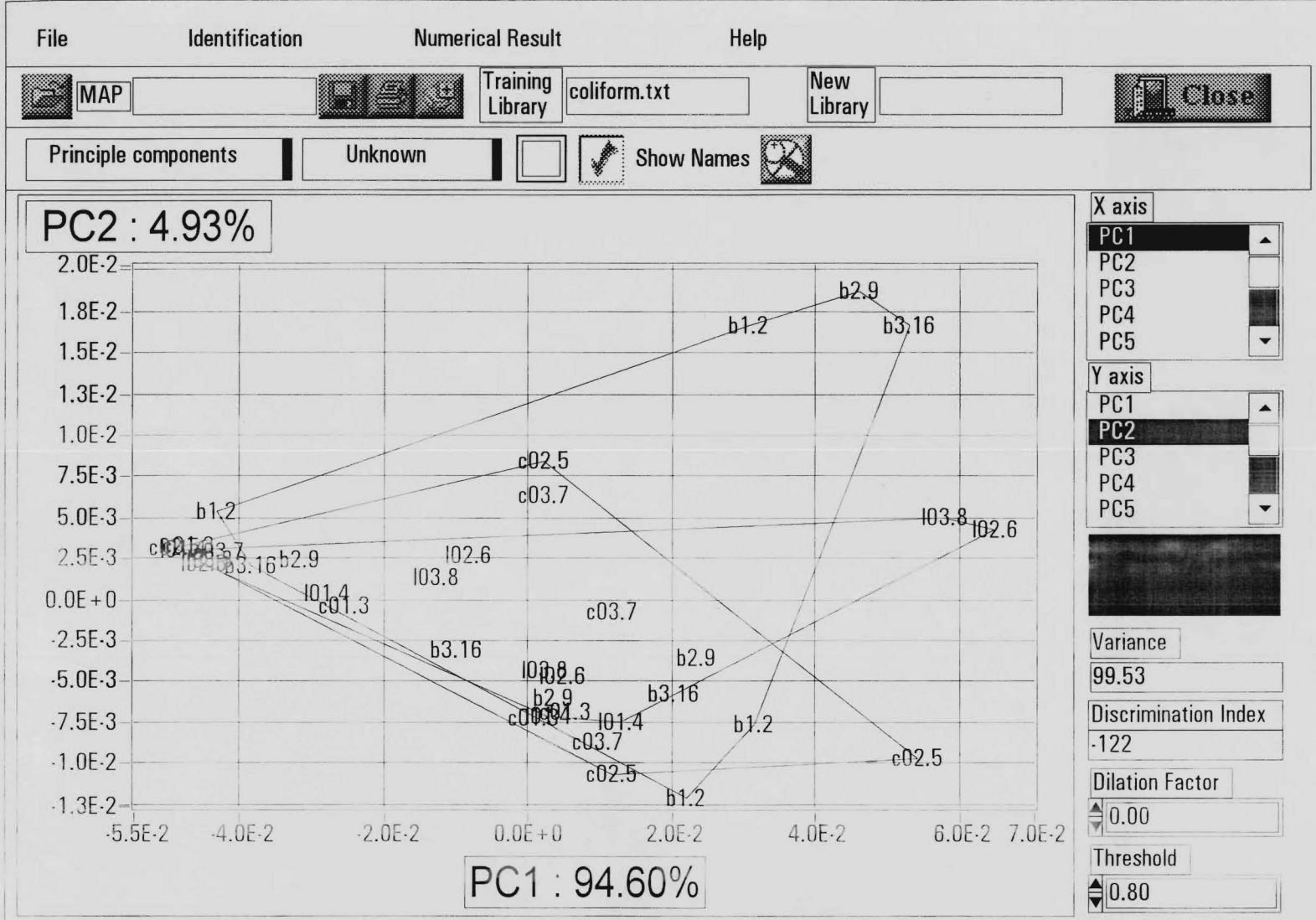
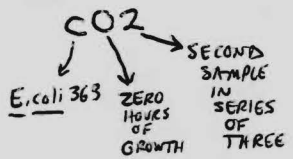


FIGURE 5, PRINCIPLE COMPONENT ANALYSIS OF E. coli 363 AND E. liquefaciens AT ZERO HOURS OF GROWTH.

- c = E. coli 363
- l = E. liquefaciens
- b = TRYPTOSE BROTH

Ex.



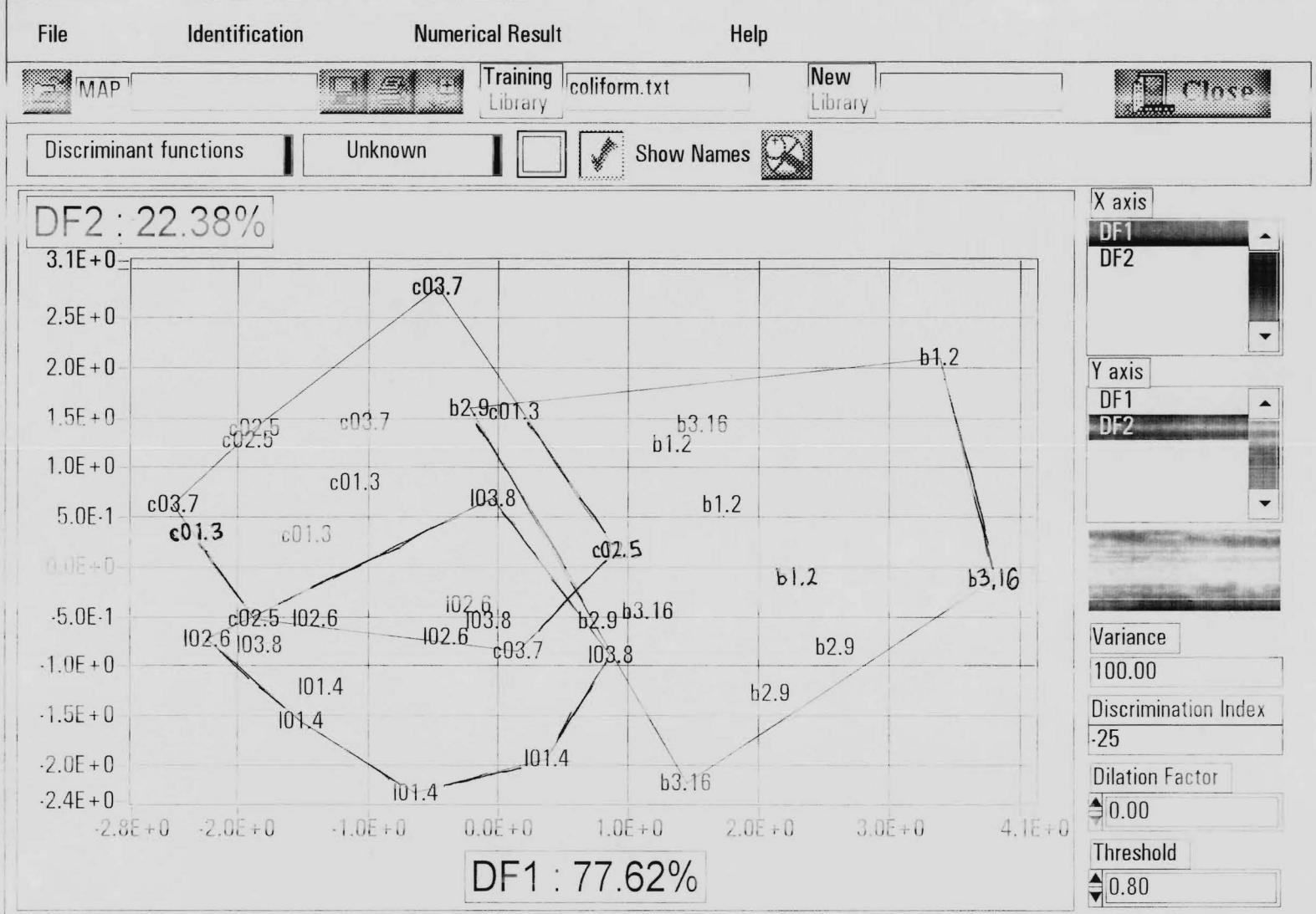


FIGURE 6. DISCRIMINANT FUNCTION ANALYSIS OF E. coli 363 AND E. liquefaciens AT ZERO HOURS OF GROWTH.

- C = E. coli 363
- I = E. liquefaciens
- b = TRYPTOSE BROTH

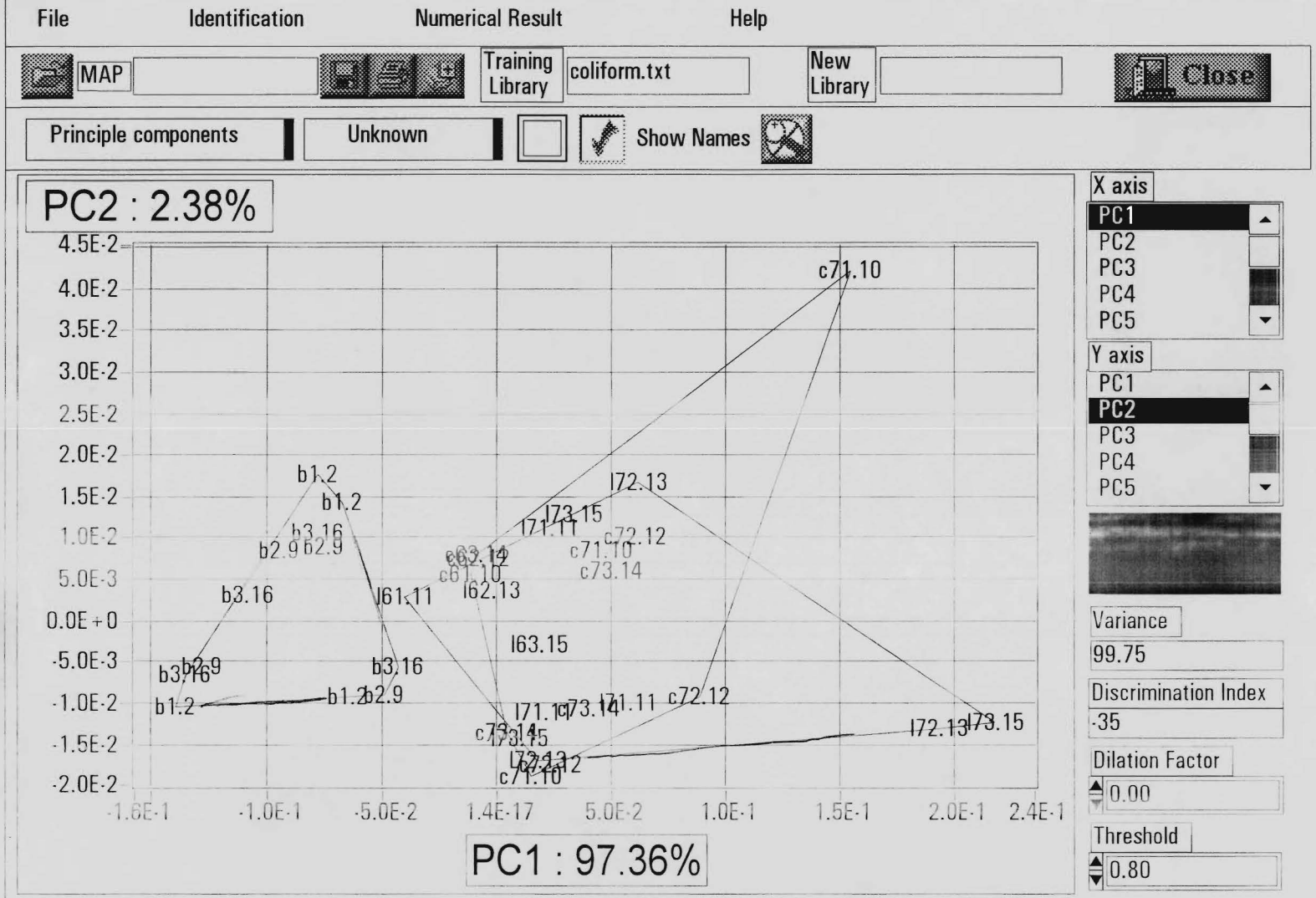


FIGURE 7. PRINCIPLE COMPONENT ANALYSIS OF E. coli 363 AND E. liquefaciens AT SEVEN HOURS OF GROWTH.

- c = E. coli 363
- l = E. liquefaciens
- b = TRYPTOSE BROTH

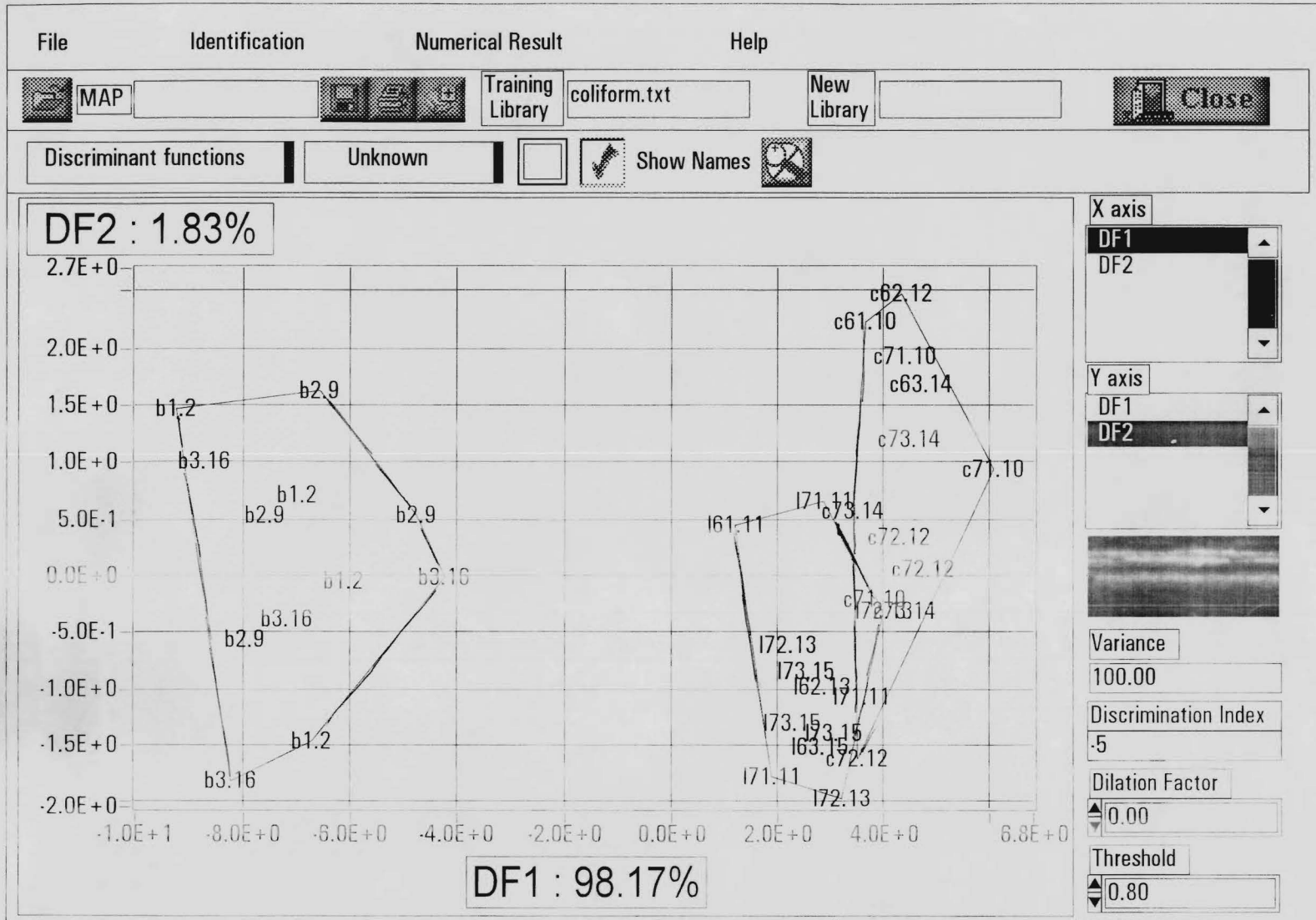


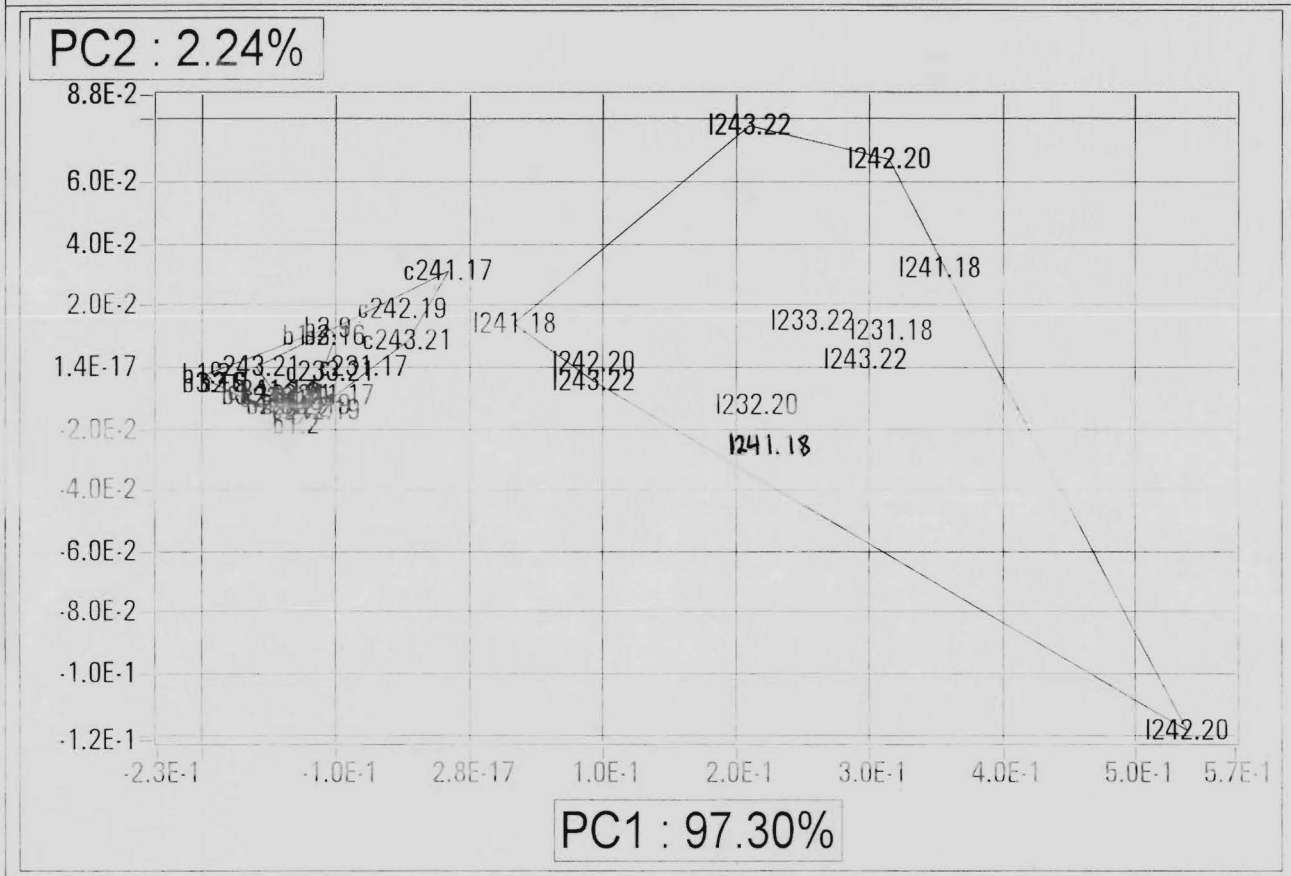
FIGURE 8. DISCRIMINANT FUNCTION ANALYSIS OF E. coli 363 AND E. liquefaciens AT SEVEN HOURS OF GROWTH.

- c = E. coli 363
- l = E. liquefaciens
- b = TRYPTOSE BROTH

File Identification Numerical Result Help

MAP | Training Library: coliform.txt | New Library | Close

Principle components | Unknown | Show Names



X axis: PC1 | PC2 | PC3 | PC4 | PC5

Y axis: PC1 | PC2 | PC3 | PC4 | PC5

Variance: 99.53

Discrimination Index: -31

Dilation Factor: 0.00

Threshold: 0.80

FIGURE 9. PRINCIPLE COMPONENT ANALYSIS OF E. coli 363 AND E. liquefaciens AT 24 HOURS OF GROWTH.

- C = E. coli 363
- l = E. liquefaciens
- b = TRYPTOSE BROTH

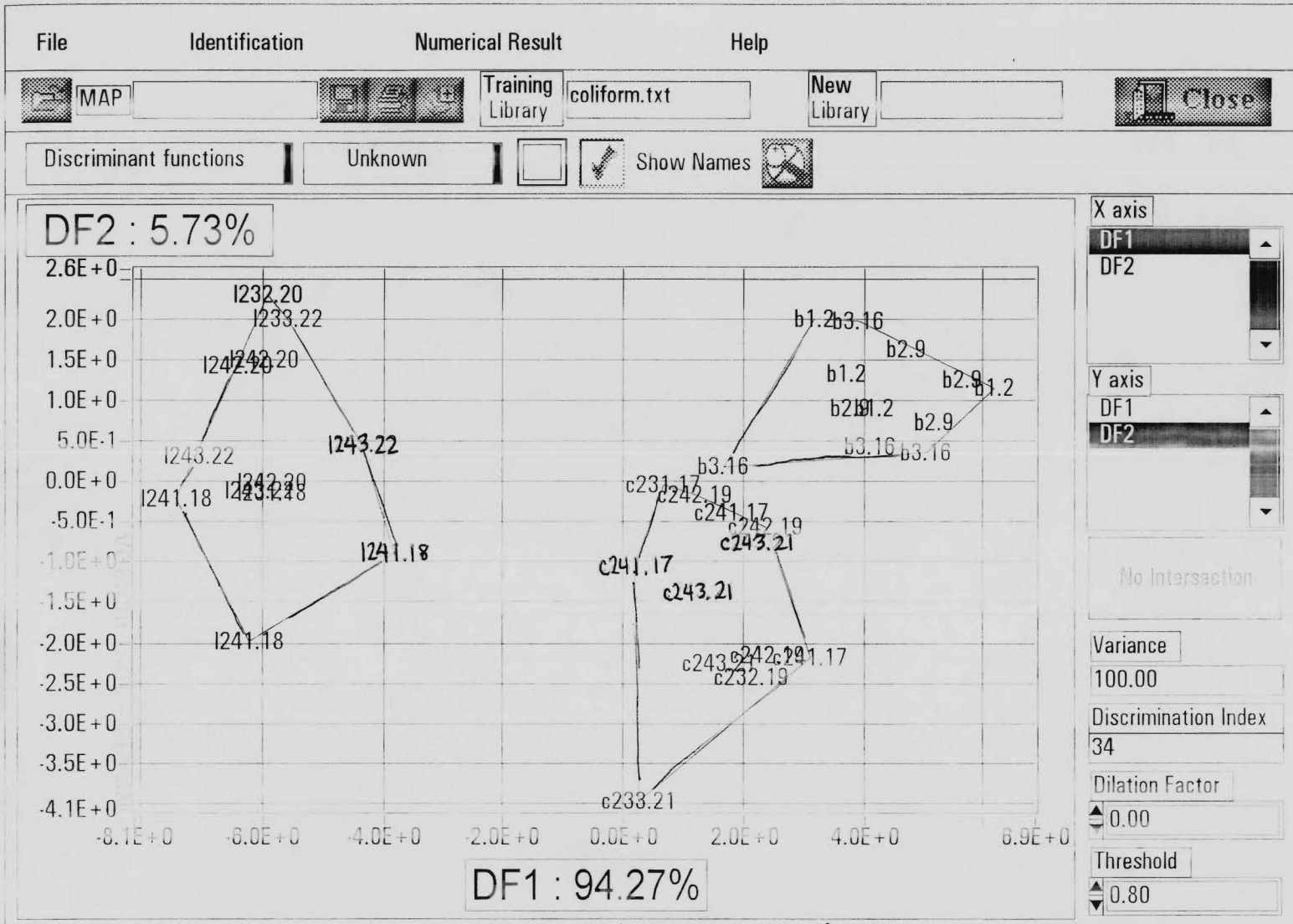


FIGURE 10. DISCRIMINANT FUNCTION ANALYSIS OF E. coli 363 AND E. liquefaciens AT 24 HOURS OF GROWTH.

- c = E. coli 363
- l = E. liquefaciens
- b = TRYPTOSE BROTH